British Journal of Pharmacology (2002) 136, 641–644

www.nature.com/bjp

SPECIAL REPORT

M_3 muscarinic receptors mediate contraction of human urinary bladder

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Since muscarinic receptors appear to be the physiologically most important control system for urinary bladder contraction, we have characterized the receptor subtype mediating contraction in response to the muscarinic agonist carbachol in the human bladder. Experiments were based on four antagonists, the non-selective atropine, the M_1-selective pirenzepine, the M_2-selective methoctramine and the M_3-selective darifenacin. All antagonists yielded Schild-plots with a slope close to unity. The order of potency (atropine $\geqslant$ darifenacin > pirenzepine > methoctramine) as well as the estimated antagonist affinities suggested that contraction of the human bladder occurs predominantly if not exclusively *via* the M_3 receptor.

British Journal of Pharmacology (2002) **136,** 641–644

Keywords: Bladder; contraction; M_3 receptor; human

Abbreviations: E_{max}, maximum response; pEC_{50}, −log of agonist concentration causing half-maximal effects

Introduction Overactive bladder is a highly prevalent disease state which is present in 17% of the general population aged 40 and over (Milsom *et al*., 2001). It markedly impairs the quality of life of the afflicted patient and places a large socio-economic burden on society (Wyndaele, 2001). Clinically overactive bladder is characterized by the symptoms of frequency (eight or more micturitions per day), urgency and urge incontinence, which occur singly or in combination, and are not explained by metabolic or local pathological factors (Wyndaele, 2001). The symptoms of overactive bladder are thought to result from involuntary bladder contractions during the filling phase of the micturition cycle (Wyndaele, 2001). Muscarinic acetylcholine receptors are the predominant receptor system controlling bladder contractility (Andersson, 1993), and muscarinic receptor antagonists are the mainstay of medical treatment for overactive bladder (Wyndaele, 2001).

Human bladder tissue contains M_2 and M_3 muscarinic receptors in 75–80 : 20–25% ratio as detected by competition radioligand binding (Goepel *et al*., 1998) or immunoprecipitation with subtype-selective antisera (Wang *et al*., 1995). A similar situation is found in the bladder of rats, rabbits, guinea-pigs (Wang *et al*., 1995) and pigs (Goepel *et al*., 1998; Yamanishi *et al*., 2000). The contractile response to the exogenous muscarinic agonist carbachol and to endogenous agonist released by field stimulation, however, occurs predominantly if not exclusively *via* M_3 receptors in rats (Choppin *et al*., 1998; Hegde *et al*., 1997; Longhurst *et al*., 1995; Longhurst & Levendusky, 2000; Tong *et al*., 1997), mice (Choppin & Eglen, 2001b), pigs (Yamanishi *et al*., 2000) and dogs (Choppin & Eglen, 2001a). Moreover, M_3 (but not M_2) receptor knock-out mice exhibit bladder distension and develop urinary retention (Matsui *et al*., 2000). The muscarinic receptor subtype mediating contraction of the human bladder has not yet been identified, but its characterization appears important to further the understanding of bladder function and dysfunction and to clarify optimal targets for drug development.

Methods *Force of contraction* Human tissue specimens were obtained from patients undergoing cystectomy due to bladder cancer. All patients had given informed written consent in accordance with the approval by the local ethical committee. The tissue samples were from tumour-free parts of the bladder. While it cannot be excluded that this tissue source has affected the receptor identification, it should be noted that our previous finding of a dominating population of M_2 receptors in the human bladder in radioligand binding assays had been generated with similar tissue samples (Goepel *et al*., 1998).

Bladder strips of approximately 10 mm length and 1–2 mm diameter were prepared and mounted in 10 ml organ baths containing Krebs–Henseleit solution ((mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 2.5, EDTA 0.027, glucose 5.5 and HEPES 10) which was aereated with 95% O_2 and 5% CO_2 to yield a pH of 7.4 at 37°C. After 60 min of equilibration, including washes with fresh buffer every 15 min, the bladder strips were challenged three times with a combination of 50 mM KCl and 0.1 mM carbachol with 5 min rest and washes between each challenge. Following washout and an additional 30 min of equilibration, cumulative concentration–response curves were constructed for carbachol. Using washout and 15 min equilibration periods in between, up to five consecutive curves were generated in the presence of increasing concentrations of the indicated antagonists.

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Chemicals Darifenacin hydrobromide was a gift from Pfizer Central Research (Sandwich, Kent, U.K.) and dissolved at 10 mM in dimethylsulphoxide. Atropine hemisulfate, carbachol hydrochloride, methoctramine tetrahydrochloride and pirenzepine dihydrochloride were from Sigma (Deisenhofen, Germany) and dissolved at 10 mM in water.

Data analysis Concentration–response experiments were analysed by fitting sigmoidal curves to the data. In these fits the bottom of each curve was set at 0, and the maximum response (E_{max}), the −log of the agonist concentration causing half-maximum effects (pEC_{50}) and the Hill-slope were calculated. Effects in the presence of the antagonists were compared to those in their absence in time control experiments. Antagonist potency was estimated by the method of Arunlakshana & Schild (1959); if the slope of the Schild regression line did not significantly differ from unity and if the antagonist did not alter maximum responses, the x-axis intercept was assumed to represent the pK_B value of the antagonist. When maximum responses were significantly affected by the antagonist, the x-axis intercept was interpreted as an apparent pA_2 value. Statistical significance of differences between groups was determined by one-way analysis of variance followed by Dunnett's multiple comparison test. All curve fitting procedures and statistical calculations were performed by the Prism computer program (GraphPAD Software, San Diego, CA, U.S.A.) and a $P<0.05$ was considered significant. The x-axis intercepts of the Schild plots (pK_B or apparent pA_2 values) are given as means with 95% confidence intervals; all other data are shown as mean±s.e.mean of n experiments.

Results The first carbachol concentration–response curve in each preparation yielded a mean pEC_{50} of 6.24±0.05 and a maximum response of 36.6±3.6 mN or 3.7±0.4 mN (mg wet weight)$^{-1}$ ($n=44$). Consecutive additional carbachol concentration–response curves within these preparations in the absence of antagonists yielded a small but gradual decline of maximum effects (Table 1) and potency of the agonist (−0.08±0.07, −0.23±0.05, −0.03±0.07 and −0.36±0.09 log units for the second, third, fourth and fifth curve, respectively; $n=14$). These shifts of the carbachol concentration–response curve during the time control experiments were taken into account when determining the antagonist-induced shifts.

The non-selective antagonist atropine, the M_1-selective pirenzepine, the M_2-selective methoctramine and the M_3-selective darifenacin concentration-dependently shifted the carbachol concentration–response curve to the right towards higher concentrations. While atropine, pirenzepine and methoctramine did not significantly alter maximum carbachol responses, darifenacin exposure significantly reduced maximum contractile effects (Table 1). Analysis of the shifts of the carbachol concentration–response curves by all four antagonists yielded Schild regression lines which were close to unity (Figure 1). From the resulting x-axis intercepts pK_B values (apparent pA_2 value for darifenacin) of 8.92 (95% confidence interval 8.62–9.27), 6.76 (6.55–7.01), 6.37 (5.98–6.90) and 8.44 (7.88–9.35) were calculated for atropine, pirenzepine, methoctramine and darifenacin, respectively.

Discussion In the present study, the muscarinic receptor subtype mediating contraction in response to carbachol in the human bladder was characterized functionally based on the non-selective antagonist atropine, the M_1-selective pirenzepine, the M_2-selective methoctramine and the M_3-selective darifenacin. While atropine, pirenzepine and methoctramine did not significantly affect the maximum response to carbachol, darifenacin significantly reduced the maximum carbachol responses. Similar unsurmountable darifenacin effects on bladder contraction have previously been described in rat (Hegde *et al*., 1997) and canine bladder (Choppin & Eglen, 2001a), but the underlying reasons are unknown.

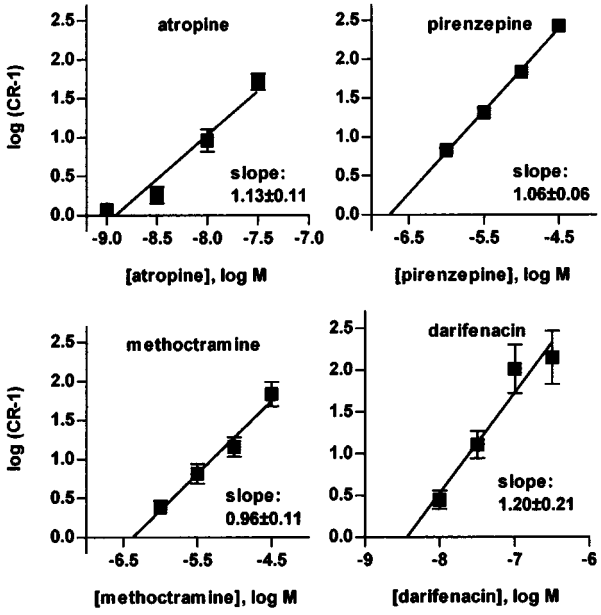

Figure 1 Schild-plots for antagonism of carbachol-induced human bladder contraction by atropine, pirenzepine, methoctramine and darifenacin. Each data point represents the means±s.e.means of 6–7 experiments.

Table 1 E_{max} of repeated carbachol-response curves in the absence and presence of receptor antagonists

	Vehicle n=14	*Atropine* n=7	*Pirenzepine* n=6	*Methoctramine* n=6	*Darifenacin* n=6
2nd curve	97±3	97±3	101±3	94±3	92±5
3rd curve	96±2	86±4	98±5	91±6	77±11*
4th curve	90±3	76±6	97±7	87±10	65±10*
5th curve	85±3	67±9	74±8	74±12	53±9*

E_{max} is expressed as % of the effect in the first curve within each preparation which was always performed in the absence of any antagonist; subsequent curves were generated in the presence of increasing antagonist concentrations (see Figure 1). Data are mean±s.e.means of the indicated number of experiments. *$P<0.05$ in one-way analysis of variance followed by Dunnett's multiple comparison test.

Nevertheless analysis of the shifts of the carbachol concentration response curve yielded slopes close to unity for all four antagonists. The rank order of the antagonist potencies (atropine $\geqslant$ darifenacin > pirenzepine > methoctramine) as well as the absolute potencies were in good agreement with those at cloned M_3 receptors and those determined functionally for antagonist of contraction in rat, mouse and dog bladder (Choppin & Eglen, 2001a, b; Hegde *et al*., 1997). These data demonstrate that contraction of human bladder in response to carbachol is mediated predominantly if not exclusively by the M_3 muscarinic receptor. This situation mimics closely the previously reported findings in rats (Choppin *et al*., 1998; Hegde *et al*., 1997; Longhurst *et al*., 1995; Longhurst & Levendusky, 2000; Tong *et al*., 1997), mice (Choppin & Eglen, 2001b), pigs (Yamanishi *et al*., 2000) and dogs (Choppin & Eglen, 2001a). These data suggest that a drug for the effective treatment of overactive bladder should have high affinity for M_3 receptors. However, it should be noted that under some pathophysiological conditions such as chronic bladder denervation M_2 receptors can also contribute to bladder contractility in rats (Braverman *et al*., 1998). Whether these findings with bladder denervation are relevant to the disease state of overactive bladder, i.e. whether a useful drug for its treatment should also have high affinity at M_2 muscarinic receptors, remains to be determined from clinical studies.

M. Fleichman was a recipient of a fellowship of the Deutscher Akademischer Austauschdienst.

References

ANDERSSON, K.-E. (1993). Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. *Pharmacol. Rev.,* **45,** 253 – 308.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.,* **14,** 48 – 58.

BRAVERMAN, A.S., LUTHIN, G.R. & RUGGIERI, M.R. (1998). M_2 muscarinic receptor contributes to contraction of the denervated rat urinary bladder. *Am. J. Physiol.,* **275,** R1654 – R1660.

CHOPPIN, A. & EGLEN, R.M. (2001a). Pharmacological characterization of muscarinic receptors in dog isolated ciliary and urinary bladder smooth muscle. *Br. J. Pharmacol.,* **132,** 835 – 842.

CHOPPIN, A., & EGLEN, R.M. (2001b). Pharmacological characterization of muscarinic receptors in mouse isolated urinary bladder smooth muscle. *Br. J. Pharmacol.,* **133,** 1035 – 1040.

CHOPPIN, A., EGLEN, R.M. & HEGDE, S.S. (1998). Pharmacological characterization of muscarinic receptors in rabbit isolated iris sphincter muscle and urinary bladder smooth muscle. *Br. J. Pharmacol.,* **124,** 883 – 888.

GOEPEL, M., GRONEWALD, A., KREGE, S. & MICHEL, M.C. (1998). Muscarinic receptor subtypes in porcine detrusor: comparison with humans and regulation by bladder augmentation. *Urol. Res.,* **26,** 149 – 154.

HEGDE, S.S., CHOPPIN, A., BONHAUS, D., BRIAUD, S., LOEB, M., MOY, T.M., LOURY, D. & EGLEN, R.M. (1997). Functional role of M_2 and M_3 muscarinic receptors in the urinary bladder of rats *in vitro* and *in vivo*. *Br. J. Pharmacol.,* **120,** 1409 – 1417.

LONGHURST, P.A., LEGGETT, R.E. & BRISCOE, J.A.K. (1995). Characterization of the functional muscarinic receptors in the rat urinary bladder. *Br. J. Pharmacol.,* **116,** 2279 – 2285.

LONGHURST, P.A. & LEVENDUSKY, M. (2000). Influence of gender and the oestrous cycle on *in vitro* contractile responses of the rat urinary bladder to cholinergic stimulation. *Br. J. Pharmacol.,* **131,** 177 – 184.

MATSUI, M., MOTOMURA, D., KARASAWA, H., FUJIKAWA, T., JIANG, J., KOMIYA, Y., TAKAHASHI, S. & TAKETO, M.M. (2000). Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M_2 subtype. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 9579 – 9584.

MILSOM, I., ABRAMS, P., CARDOZO, L., ROBERTS, R.G., THÜROFF, J.W. & WEIN, A.J. (2001). How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. *BJU Int.,* **87,** 760 – 766.

TONG, Y.-C., HUNG, Y.-C., LIN, S.-N. & CHENG, J.-T. (1997). Pharmacological characterization of the muscarinic receptor subtypes responsible for the contractile response in the rat urinary bladder. *J. Auton. Pharmacol.,* **17,** 21 – 25.

WANG, P., LUTHIN, G.R. & RUGGIERI, M.R. (1995). Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. *J. Pharmacol. Exp. Ther.,* **273,** 959 – 966.

WYNDAELE, J.J. (2001). The overactive bladder. *BJU Int.,* **88,** 135 – 140.

YAMANISHI, T., CHAPPLE, C.R., YASUDA, K. & CHESS-WILLIAMS, R. (2000). The role of M_2-muscarinic receptors in mediating contraction of the pig urinary bladder *in vitro*. *Br. J. Pharmacol.,* **131,** 1482 – 1488.

(Received March 7, 2002
Revised April 8, 2002
Accepted April 24, 2002)